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Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay

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Running title: Brevibacterium marker for fecal source tracking of poulty

ABSTRACT

A poultry litter-specific biomarker was developed for microbial source tracking (MST) in environmental waters. 16S rRNA sequences that were present in fecal-contaminated turkey and chicken litter were identified by terminal restriction fragment length polymorphism (T-RFLP). Cloning and sequencing of potential targets from pools of *E. coli*, *Bacteroides* or total bacterial DNA yielded four sequences that were ubiquitous in poultry litter and also contained unique sequences for development of target-specific PCR primers. Primer sensitivity and specificity were tested by nested PCR against ten composite poultry litter samples and fecal samples from beef and dairy cattle, swine, ducks, geese, and human sewage. The sequence with greatest sensitivity (100%) and specificity (93.5%) has 98% identity to *Brevibacterium avium*, and was detected in all litter samples. It was detected at low level in only one goose and one duck sample. A quantitative PCR assay was developed and tested on litter, soil and water samples. Litter concentrations were $2.2*10^{7}$ - $2.5*10^{9}$ gene copies/g. The biomarker was present in a majority of soil and water samples collected in and near areas where litter was spread, reaching concentrations of 2.9×10^{5} gene copies$\cdot g^{-1}$ in soil samples and 5.5×10^{7} gene copies$\cdot L^{-1}$ in runoff from the edges of fields. The biomarker will contribute to quantifying the impact of fecal contamination by land-applied poultry litter in this watershed. Furthermore, it has potential for determining fecal source allocations for total maximum daily load (TMDL) programs and ambient water quality assessment, and may be useful in other geographic regions.

INTRODUCTION

Excessive land application of poultry litter as a waste disposal mechanism has been linked to eutrophication of water bodies (28, 35, 39), the spread of pathogens (15, 19, 21), air and soil pollution with metals (11, 33) and groundwater contamination with nitrate (5). Despite these known effects, land application is still the typically practiced disposal method for poultry litter even though viable and economically favorable alternative disposal practices are available (7, 20).

Identification of the source of fecal pollution contaminating a watershed is of particular interest for protection of water resources and the safety of recreational waters. For example, TMDL assessments require identification of the source of contamination, which is also necessary for remediation of impaired waters(44). Current methods for detecting the presence of fecal pollution, which carries an increased risk of the presence of pathogenic microorganisms, involve the cultivation of fecal indicator organisms such as fecal coliforms in the family *Enterobacteriaceae* (Oklahoma Administrative Code, Title 785, Chapter 46). The U.S. EPA and many states recognize *Escherichia coli* and enterococci as indicators of freshwater recreational water quality (42).

Drawbacks to the use of indicator organisms which limit the ability of researchers to pinpoint sources of fecal contamination include the non-specificity of the fecal coliforms to one source (25, 43), variable survival rates of various indicator organisms (1) and the growth or extended persistence of these indicator organisms after release to the environment (12, 45). These drawbacks have lead to research into alternative methods for the assessment of human health risk

from microbial pathogens in recreational waters that do not include the culturing of fecal indicator organisms for identification and quantification of the source of fecal pollution (46).

A variety of microbial source tracking (MST) methods (for recent reviews see (17, 40, 47)) have been proposed as an alternative to cultivation of fecal coliforms. Some of these genotypic molecular based techniques have included library dependent methods (i.e., culture and isolate-based) such as ribotyping (10, 31) and repetitive element polymerase chain reaction (REP-PCR) (14). Library independent methods (i.e., detection of a genetic biomarker in extracted DNA) have also been developed using discovery techniques such as suspension arrays (8), subtractive hybridization (13, 26), and terminal restriction fragment length polymorphism (T-RFLP) (3), among others. Host marker specific targets have included *Enterococcus faecium* (37), *Bifidobacterium* and members of the *Bacteroidales* (3, 22, 38), among others. Relatively few microbial targets specific to poultry fecal material have been identified. To date *Enterococcus faecalis* (23), *E. coli* (10) and *Bacteriodes* (26) have been associated with poultry fecal material, but only the *Bacteroides* biomarker (26) was specifically associated with poultry and not other fecal sources The objective of this research was to identify a poultry litter-specific biomarker, validate its specificity against other sources of fecal material from within and outside the watershed and develop a 16S rRNA based real-time PCR assay for quantifying the biomarker in environmental samples. This work was carried out as part of ongoing litigation in which the plaintiff is the Oklahoma Attorney General.

METHODS

Sample collection. Litter samples were collected from ten separate facilities (poultry houses), nine chicken and one turkey facility. Litter samples were collected from 18 locations within each

poultry house through the entire depth of the litter. The subsamples (total volume of 4 to 5 gallons) from each house were composited, homogenized and split (riffle splitter) before placement into a sterile whirl pack (approximately 500 mL) and shipped on ice to the laboratory for analysis. Litter application areas in fields (soils) were sampled by collecting 20 subsamples on a predetermined grid pattern across a uniform subarea of one to ten acres in size. The zero to two inch sample from six inch soil cores were composited, disaggregated, sieved to 2 mm, ground, homogenized and split. Vegetation, feathers, and rocks were removed. The split soil samples (500 ml) were transported on ice to the laboratory. Nontarget fecal samples for specificity testing were collected as composites from groups of individuals (Table 3). Samples from beef cattle were collected from ten grazing fields, of which five were within the watershed and five were outside the watershed. Two independent duplicate samples were collected for each field, and each duplicate consisted of feces from ten scats. A total of 200 beef cattle scats were collected and composited into 20 samples. Duck and goose samples were collected in the same fashion, consisting of composites from ten individual scats, and independent duplicates were collected for each area. For ducks, three landing areas inside the watershed and two outside the watershed were sampled, while for geese, two landing areas inside and three landing areas outside the watershed were sampled. A total of 100 scats for duck and geese were collected and composited into 10 samples for duck and 10 samples for geese. Composite samples of fecal slurries were collected from swine facilities, one inside the watershed and one outside (2 duplicate samples) and dairy cattle facilities (one inside the watershed and two outside (2 duplicate samples each) human residential septic cleanout trucks (3 samples) and influent of three separate municipal wastewater treatment plants (3 samples). A total of 20 g of each fecal sample other than litter from each site was collected and was placed in a 20 ml, sterile,

polystyrene tube containing 10 ml of 20% glycerol and shipped on dry ice to the laboratory. All fecal samples were homogenized in the glycerol before DNA extraction. Discrete water samples from larger rivers and lakes were collected using a Van Dorn water sampler or with a churn splitter for discrete or composite samples. Samples from larger rivers were typically composites of 3 samples collected on a transect across the width of the river channel. Samples from smaller rivers were collected using automated samplers. Samples collected during high flow events were composited based on flow volume. Base flow samples were collected as grab samples. River samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Runoff samples from the litter application areas (e.g. edge of field runoff samples) were collected during or as soon as possible after rainfall events. Samples were collected either with a passive runoff collector for composite samples or with a dip sampler for discrete samples. Runoff samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Groundwater samples were collected directly from existing homeowner's wells or from hydraulically driven shallow probes. Spring samples were collected as grab samples or by using a peristaltic pump. All samples were placed into sterile 1-L polystyrene bottles and shipped onice to the laboratory where they were filtered.

Enumeration of Indicator Bacteria. Indicator bacteria (fecal coliforms, *E. coli* and enterococci) were enumerated according to standard methods using multiple tube fermentation (MTF) and calculation of the most probable number according to according to SM-9221F or SM-9230 (APHA, 2005). MTF tubes containing *E. coli* were identified using broth cultures supplemented with (MUG) (SM-9221F) (2).

Soil, Litter and Fecal Sample DNA Extraction. Genomic DNA was extracted from soil, liter and fecal samples with Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc.) following the manufacturer's instructions. Typically 0.25 g of soil or litter was used in each extraction. DNA was purified by size-exclusion chromatography. Sepharose CL-4B (Sigma-Aldrich) was resuspended in Tris-HCL and sterilized by autoclave at 121 ^{o}C for at least 20 minutes. Micro-bio spin columns (Bio-Rad Laboratories) were packed with 1 mL of Sepharose CL-4B through centrifugation. Sepharose columns were then washed twice with Tris-HCl buffer (pH 8) and 50 to 150 µl of sample was added. Purified DNA was concentrated with ethanol precipitation and re-eluted in 100 µL sterile water.

Water Sample DNA Extraction. Within 12 hours of receipt at the laboratory all water samples were filtered through a sterile Supor-200, 0.2 µM filter and frozen at -80°C. Filters were then shattered with sterile glass beads and vortexed vigorously for 15 minutes with sterile, DNase, and RNase free water to remove solids and cells from the filters. The cell suspension was removed from the centrifuge tubes by pipette and placed in a 2 mL bead beating tube from the Bio101 Fast®Spin® DNA extraction kits. The cells were centrifuged at 20,000 x g for 10 minutes, and the supernatant was decanted. Genomic DNA was then extracted using the Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc). The extracted DNA was quantified using a Nanodrop® UV-Vis Spectrophotometer.

T-RFLP Analysis. Extracted genomic DNA and/or cloned DNA was amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R (16, 24), with *E.coli* genus specific primers (Tsen, et al. 1998), and *Bacteroidales* specific primers (Bernhard and Field, 2000). All PCR primers targeted the 16S rRNA gene. Triplicate PCR reactions were generated from each DNA extraction, combined and purified

using QIAquick PCR purification Kits (Qiagen). Approximately 200 ng each of PCR product was digested at 37°C for 6 hours with the *Msp*I restriction enzyme (20µ/µL) (New England BioLabs). Samples were denatured by heating to 95° C for 3 minutes followed by cooling to 4°C. The digested fragments were purified by ethanol precipitation.

Primer Design. Primers were designed using the ABI Primer Express v.2 program (Applied Biosystems, Foster City, CA) and were targeted to variable regions between the potential biomarker sequences and sequences of the top 20 closest related organisms in the GenBank database. The BLAST search (Basic Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to check the specificity of each primer.

PCR Assay Conditions. PCR was used to amplify approximately 900 bp of the 16S rRNA genes from *Bacteria* for clone library construction. Each 25 µL PCR reaction included 0.4 mg mL^{-1} molecular-grade bovine serum albumin (BSA) (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5 µM of both the forward (8F) (16) and reverse (907R) (24) primer (Invitrogen), 1U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1 µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocycler using the following conditions: 94 °C for 5 minutes, 30 cycles of 94 °C (1 minute), 55 °C (45 seconds), and 72 °C (2 minute). A final extension at 72 °C for 7 minutes was performed and the PCR products were held at 4°C. Specificity of the PCR primers to the poultry litter biomarker was evaluated with nested PCR by first amplifying non-target fecal samples by universal bacterial primers 8F, 907R and then amplifying by the potential poultry litter biomarker PCR primers. The nested PCR master mix and thermocycler conditions were similar to the universal PCR with the following exceptions: 1) forward and reverse PCR

primers were specific to the potential poultry biomarker as shown in Table 2, 2) the annealing temperature was 60 ^{o}C. Amplification by nested PCR was evaluated by gel electrophoresis.

Clone Libraries. Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial primers 8F-907R (16, 24), targeting the 16S rRNA genes of *Bacteria* or the *E. coli* genus specific primers V1SF-V3AR (41). The TOPO ® Cloning Reaction methods from Invitrogen ^{TM} were followed for clone library construction. Two clone libraries were constructed (targeting *Bacteria* and *E. coli*) from pooled DNA samples (i.e., 1 µl of genomic DNA extract from each sample was added to the PCR reaction for inclusion into the clones) based on the abundance of the various potential biomarkers as evidenced by the T-RFLP profiles.

qPCR Assay Conditions. Quantitative PCR (qPCR) was used to amplify 530 bp of the 16S rRNA gene from *Brevibacterium spp*. DNA samples were diluted to final concentrations of 3 ng/µL DNA. Each 25µL qPCR reaction included: 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward (LA35F) and reverse primer (LA35R) (Invitrogen), 5 % DMSO, 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50 ^{o}C for 2 minutes, 95 ^{o}C for 15 minutes, 45 cycles of 95 ^{o}C (30 seconds), 60 ^{o}C (30 seconds), and 72 ^{o}C (30 seconds) with a plate read. The 45 cycles was followed by a final extension at 50 ^{o}C for 5 minutes. Immediately following the final extension was a melting curve from 70 ^{o}C to 90 ^{o}C, by 0.1 degree increments, holding for 5 seconds with a plate read. DNA standards ranging from $6*10^{-15}$ to 10^{-21} ng/ul were prepared from serial dilutions of clone plasmid DNA containing the sequence of interest and used to develop the standard curve and method detection limit. Gene copy numbers were calculated from concentrations of positive control standards assuming 9.124

* 10^{14} bp/ul of DNA and one gene copy per genome. Detection limits for the qPCR assay were approximately 2000 plasmid copies in *E. coli*/L water and 7.3 *10^4 plasmid copies in *E. coli*/gram of soil. Nested qPCR was performed by first amplifying DNA with the universal bacterial 16S rRNA 8F (16) and 907R (24) primers. The production of PCR products was confirmed on a 1.5% agarose gel. The 16S rRNA PCR products were purified with the QIAquick PCR purification kit (QIAGEN) were subjected to qPCR as previously described using the LA35F and LA35R primers for the poultry litter biomarker.

Phylogeny. The phylogeny of the LA35 clone was investigated using the following methods. The clone sequences were assembled and aligned with BioEdit v. 7.0.5.3 and sequences were checked for chimeras with the Ribosomal Database Project II Chimera Check program and Bellerophon. The 16S rRNA sequences of the closest neighbors to the clone sequences were downloaded for inclusion in the phylogenic analysis. Multiple sequence alignments were constructed with Clustal W alignment tool and manually aligned in BioEdit. The bootstraps (1000 resamplings), maximum likelihood and distance matrix analysis (Kimura), and the reconstruction of the phylogenetic trees (FITCH) were performed with the Phylip 3.65 package and in particular the programs SEQBOOT, DNAML, DNADIST, FITCH, CONSENSE, and RETREE. The reconstructed phylogenetic tree was visualized with PhyloDraw V. 0.8 (Graphics Application Lab, Pusan National University).

RESULTS

Identification of potential biomarkers by T-RFLP. A total of 20 T-RFLP profiles were generated from the 5 subsamples of each of the two litter and two soil samples. The T-RFs common among the subsamples and representing more than 1% of the community were selected

for cloning and sequencing (Table 1). A total of 3 *E. coli* T-RFs (i.e., T-RF 496.0, 498.9 and 500.8) and 3 *Bacteria* T-RFs (i.e., T-RF142.9, 147.3 and 158.9) were selected for cloning and sequencing. Clone libraries were constructed from PCR products amplified with *E. coli* specific primers (V1SF-V3AR) (41) or universal bacterial primers (8F-907R) (16, 24). A total of 300 plasmids from the clone libraries were randomly picked. T-RFLP analysis was carried out on each plasmid insert to identify which plasmids contained the T-RFs of potential biomarkers. Inserts containing the T-RFs of interest were sequenced and PCR primers were developed for those sequences containing mismatches as compared to BLAST database results of the top 20 closely related organisms. In all 4 PCR primers for members of 4 genera were developed; a *Brevibacterium spp*., a *Rhodoplanes spp*., a *Kineococcus spp*. and a *Pantoea ananatis* strain (Table 2). Two *E. coli* T-RFs were from plasmids that did not contain mismatches between the sequence of interest and the sequences of closely related organisms identified in a BLAST search and therefore were not appropriate biomarkers.

Evaluation of biomarkers against fecal samples. The PCR assays developed for the 4 potential biomarkers of poultry litter were tested for amplification against a variety of nontarget fecal samples from within and outside the watershed (Table 3). Only the *Brevibacterium* clone LA35 appeared to be a potential candidate biomarker for poultry litter in that did not amplify in any fecal samples with the exception of weak amplification in one duck and one goose sample from outside the watershed when analyzed with a nested PCR approach (i.e. PCR with universal bacterial primers and then with the *Brevibacterium* clone LA35 primers). The reconstructed phylogenetic tree of the *Brevibacterium* clone LA35 in relationship to other *Brevibacterium* spp. is presented in Figure 1.

Quantification of the poultry litter biomarker in environmental samples. A SYBR green qPCR protocol was developed and optimized using the LA35F and LA35R primers (Table 2) specific to the *Brevibacterium* clone LA35 poultry litter biomarker. The standard curve of the qPCR assay for the biomarker is presented in Figure 2. The detection limit of the qPCR assay was 6 gene copies/ul of extracted DNA.

Environmental samples from the potential poultry litter impacted watershed were tested for the presence of the biomarker with the qPCR assay (Table 4). A variety of samples from within the watershed were tested, some of which were expected to contain the biomarker (e.g., litter, contaminated soil, runoff samples), some of which had variable potential for higher biomarker levels (e.g., surface water), and some of which had lower potential for biomarker presence (i.e., groundwater samples).

The correlation between the poultry litter biomarker concentration (i.e., as quantified by qPCR) in water and litter samples and *E. coli* and *Enterococcus* as measured by most probable number is presented in Figures 3 and 4. In general the *Enterococcus* MPN counts were well correlated with the concentration of the biomarker in litter ($R^2 = 0.75$) and with the biomarker concentration in water samples ($R^2 = 0.89$). The correlation between *E. coli* concentrations and the biomarker in water samples was also strong ($R^2 = 0.85$) while *E. coli* was less tightly (but significantly) correlated with the biomarker in litter samples ($R^2 = 0.28$). Correlation of the biomarker with *E. coli* and *Enterococcous* spp. provides a line of evidence of the human health risk associated with the runoff from poultry litter application to fields although there is evidence that regrowth of these organisms is possible once they are introduced into the environment (36).

DISCUSSION

The *Brevibacterium* sp. poultry litter biomarker developed in this study was validated in terms of sensitivity (100%) against numerous positive (poultry litter) samples from different locations with the watershed and for specificity (93.5%) against composite non-target fecal samples. These practices are in accordance with recent critical reviews (34, 40) that strongly recommend MST method validation. Future efforts will attempt to extend the method validation outside the watershed and possible outside the region as this biomarker could be useful for identifying fecal pollution sources in other river systems and coastal waters.

The *Brevibacterium* clone LA35 poultry litter biomarker was most closely related to *Brevibacterium avium*, which is associated with bumble-foot lesions in poultry (32). *Brevibacterium* spp. were recently identified in spent mushroom compost that was originally derived from chicken litter and cereal straw (29). Additionally *Brevibacterium avium*, *Brevibacterium iodinum*, and *Brevibacterium epidermidis* were found to represent more than 7% of a 16S rRNA clone library originating from broiler chicken litter (27). Certain *Brevibacterium* spp. are associated with milk and cheese curds(6), human skin(9), and soils (30). *Brevibacterium* spp. have been associated with disease in humans although to date these opportunistic pathogens have only been isolated from immunocompromised patients (4, 9, 18).

As poultry litter is land-applied as a disposal practice (19, 33, 35), it was important to identify a marker that could survive the process of deposition on bedding and spreading on fields. Therefore, the T-RFLP screening process included both litter and contaminated soil samples. This strategy allowed for the rapid elimination of numerous targets that could be abundant in the poultry fecal material, but not as abundant in the litter and not present in the environment after

277 litter application. This strategy for marker identification is in contrast with the work by Lu and
278 colleagues (2007) where a genome fragment enrichment method was used to identify microbial
279 sequences specific to chicken feces. Based on the PCR assays developed from clone libraries of
280 the genome fragments, 6 to 40% of the chicken fecal samples collected from a wide geographic
281 region contained DNA that could be amplified by the various assays (26). In comparison the
282 LA35 biomarker was found in all the poultry litter samples tested, although it should be noted
283 that all of the samples were collected in the Oklahoma/Arkansas region.

284 The examination of environmental samples from within the poultry litter impacted watershed
285 suggest a correlation between the application of poultry litter to a field and concentration of the
286 biomarker in the receiving waters, as evidenced by the generally decreasing trend in biomarker
287 concentration with decreasing concentration of fecal indicator organisms. These results indicate
288 that the watershed is in fact being impacted by the application of poultry litter to fields within the
289 watershed. However, the magnitude of the impact as measured by the distribution of the
290 biomarker within the watershed cannot be quantified with the limited number of environmental
291 samples processed to date. Future work will include the testing of environmental samples from
292 within the watershed by the qPCR assay to evaluate the distribution of the poultry litter-specific
293 biomarker as compared to indicator bacteria, antibiotics and heavy metals. Additionally, testing
294 of the poultry litter-specific biomarker against more fecal samples from other watersheds and
295 additional avian fecal material will be conducted as the LA35 poultry litter biomarker was found
296 in low abundance (i.e., a nested PCR approach was required for detection) in two non-target
297 composite avian fecal samples (i.e., a duck and a goose sample) from outside the watershed.

298 **Conclusions**

In summary a novel biomarker of poultry litter was identified and a 16S rRNA based real-time PCR assay was developed for this biomarker. The specificity of the assay (93.5%) was tested against 31 separate non-target fecal samples and sensitivity was tested against10 target litter samples (100%). The field applicability of the assay was evaluated by testing for the biomarker in environmental samples expected to have variable concentrations of the biomarker, which we hypothesized would be correlated with the concentration of fecal indicator bacteria. A generally positive correlation was found between biomarker concentration and fecal indicator bacteria concentration which was particularly strong for enterococci. The research presented herein is the first identification of a *Brevibacterium* spp. for microbial source tracking studies and is among the first quantifiable method for tracking of poultry fecal sources in environmental waters.

ACKNOWLEDGMENTS

This research was conducted in connection with work performed as retained experts in a pending legal case brought by the State of Oklahoma against several poultry integrators. Drs. Harwood & Olsen have been retained to serve as expert witnesses by the State of Oklahoma and have provided testimony regarding this research.

The authors are grateful for the assistance provided by Kyle Collins, William Blackmore, James Jackson, Erin O'Leary Jeapson and Michelle Andrews. Additionally the authors acknowledge the Molecular Research Core Facility at Idaho State University for graciously allowing us the use of their laboratory space and equipment.

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47. **Wuertz, S., and J. Field.** 2007. Emerging microbial and chemical source tracking techniques to identify origins of fecal contamination in waterways. Water Research **41:**3515-3516.

454 Table 1. Common T-RFs among replicates from two fecal-contaminated poulty litter samples
455 and two soils to which the litter had been applied.
456

	Number of subsamples tested (number containing T-RF of interest)			
T-RF	Litter A	Litter B	Soil A	Soil B
E.coli PCR products, digested with *MspI*				
496.0	4 (4)	5 (4)	5 (3)	5 (5)
498.9	4 (4)	5 (5)	5 (4)	5 (5)
500.8	4 (4)	5 (5)	5 (5)	5 (5)
Universal bacteria PCR products, digested with *MspI*				
80.1	4 (4)	5 (5)	5 (0)	3 (3)
130.9	4 (3)	5 (5)	5 (1)	3 (0)
142.9	4 (4)	5 (4)	5 (2)	3 (2)
147.3	4 (4)	5 (5)	5 (5)	3 (2)
158.9	4 (3)	5 (5)	5 (4)	3 (2)
165.0	4 (3)	5 (5)	5 (4)	3 (2)

*Underlined T-RFs correlate to those organisms for which PCR primers were developed

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459 Table 2. Nucleotide sequences and targets of primers used in this study.

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Primer	Target	Sequence (5'-3')	Position	Tm (°C)	T-RF
LA35F	*Brevibacterium*	ACCGGATACGACCATCTGC	166-184	57	147.3
LA35R	clone LA35	TCCCCAGTGTCAGTCACAGC	717-736	58	
SA19F	*Kineococcus*	TACGACTCACCTCGGCATC	163-181	56	158.9
SA19R	*spp.*	ACTCTAGTGTGCCCGTACCC	602-621	55	
SB37F	*Rhodoplanes*	AACGTGCCTTTTGGTTCG	143-160	56	142.9
SB37R	*spp.*	GCTCCTCAGTATCAAAGGCAG	616-626	55	
SA15F	*Pantoea*	CGATGTGGTTAATAACCGCAT	490-510	56	500.8
SA15R	*ananatis*	AAGCCTGCCAGTTTCAAATAC	668-688	55	

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462 Table 3. Specificity of the poultry litter biomarker assay tested against fecal samples from within and outside the watershed.

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	Number of samples tested (Number of samples containing potential biomarker)			
Fecal sample (inside or outside watershed)	*Brevibacterium* clone LA35	*Rhodoplanes* clone SB37	*Kineococcus* clone SA19	*Pantoea ananatis* clone SA15
Beef cattle (outside)	5 (0)	5 (2)	5 (1)	5 (0)
Beef cattle (inside)	5 (0)	5 (3)	5 (5)	5 (1)
Dairy cattle (outside)	2 (0)	2 (1)	2 (1)	2 (1)
Dairy cattle (inside)	1 (0)	1 (1)	1 (0)	1 (0)
Swine (outside)	1 (0)	1 (1)	1 (1)	1 (0)
Swine (inside)	1 (0)	1 (0)	1 (0)	1 (0)
Duck (outside)	2 (1)*	2 (2)	2 (2)	2 (2)
Duck (inside)	3 (0)	3 (1)	3 (1)	3 (2)
Goose (outside)	3 (1)*	3 (3)	3 (2)	3 (2)
Goose (inside)	2 (0)	2 (2)	2 (1)	2 (1)
Human sewage (outside)	2 (0)	2 (2)	2 (2)	2 (1)
Human sewage (inside)	4 (0)	4 (3)	4 (1)	4 (1)

* One duplicate amplified when analyzed with a nested PCR assay.

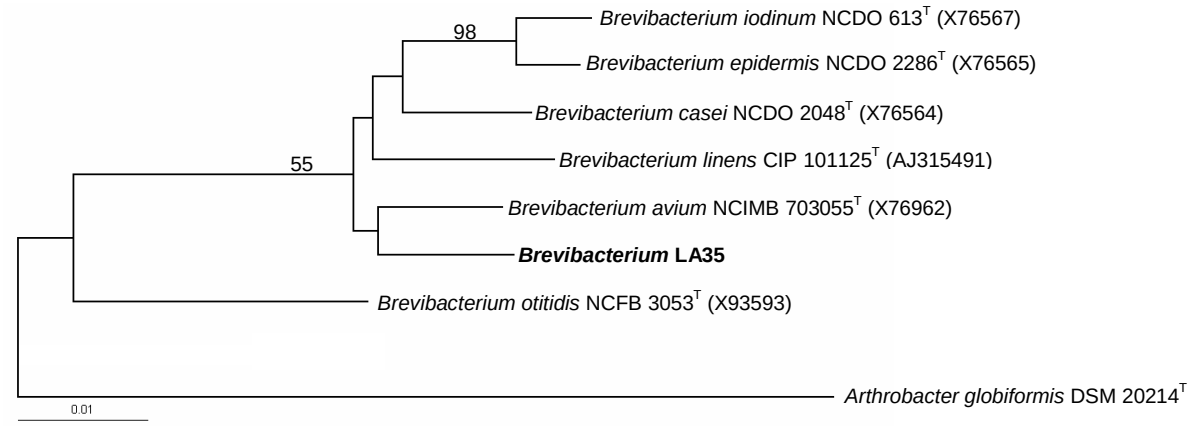

Figure 1. Reconstructed phylogentic tree of the *Brevibacterium* spp. based on 16S rRNA. Numbers at the nodes represent bootstrap values (i.e. the number of times this organism was found in this position relative to other organisms in 1000 resamplings of the data). Bootstraps less than 50% are not shown. The closest cultured organisms as reported in an NCBI BLAST search are reported. The distance bar represents a 1% estimated sequence divergence.

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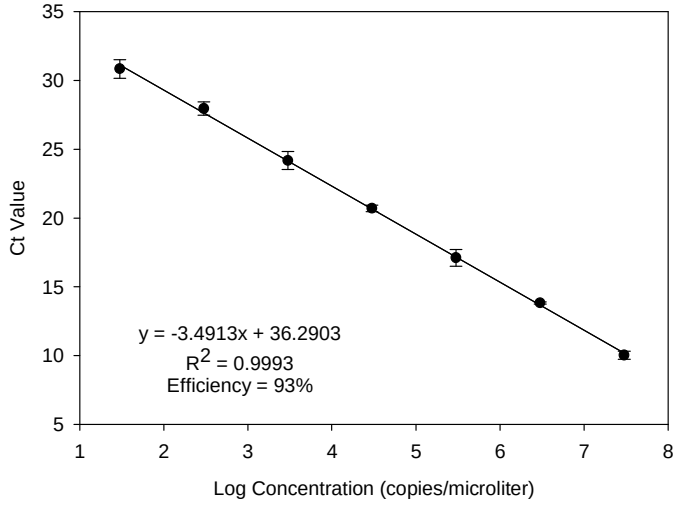

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477 Figure 2. Standard curve of measured Ct values and standard deviations versus log plasmid

478 biomarker concentration.

479 Table 4. Environmental samples tested for *Brevibacterium* clone LA35 poultry litter biomarker

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Sample type	Number samples tested	% of samples containing biomarker [a]	% of samples quantifiable [b]	Range of biomarker present (16S rRNA copies/L water or g soil or g litter)
Litter	10	100	100	$2.2*10^7 \pm 7.1*10^6 – 2.5*10^9 \pm 9.5*10^7$
Soil	10	100	50	$7.0*10^3 \pm 4.4*10^2 – 2.9*10^5 \pm 2.0*10^4$
Edge of field runoff	10	100	100	$2.6*10^3 \pm 1.2*10^2 – 5.5*10^7 \pm 5.3*10^6$
River	10	50	20	$2.9*10^3 \pm 8.6*10^2 – 3.2*10^4 \pm 6.8*10^3$
Groundwater	6	0	0	Not applicable

[a] indicates the percent of samples in which the biomarker was identified by qPCR or nested qPCR methods

[b] indicates the percent of samples for which a quantifiable number of biomarker genes were measured by qPCR

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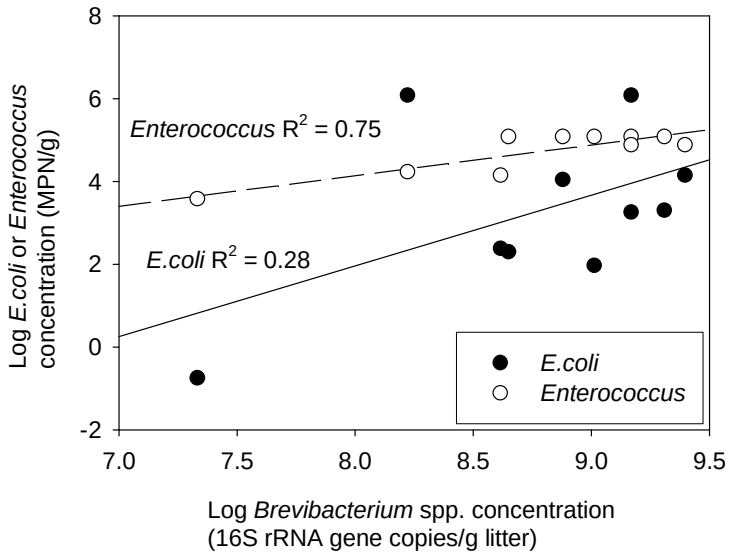

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484 Figure 3. Correlation between the concentrations of poultry litter biomarker, *E. coli* and

485 *Enterococcus spp.* in poulty litter samples.

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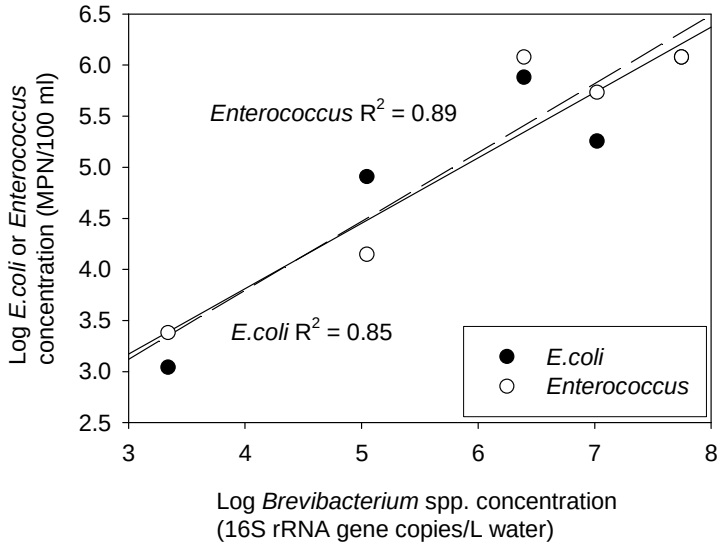

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489 Figure 4. Correlation between the concentrations of poultry litter biomarker, *E. coli* and

490 *Enterococcus* spp. in water samples.

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